Model legumes contribute to faba bean breeding

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Abstract

Faba bean is an excellent candidate crop to provide nitrogen input into temperate agricultural systems. However, its growth is hampered by several factors including environmental stresses and the presence of anti-nutritional factors. To solve these limitations, breeding programs have been initiated that were successful for monogenic traits but not so for multigenic traits. The large genome size of faba bean has slowed down breeding processes. Several other legumes have emerged as model legumes including *Medicago truncatula, Lotus japonicus, Glycine max* and *Pisum sativum*. The establishment of these models has already boosted our understanding of important processes such as the nitrogen-fixing symbiotic interaction. The high level of synteny and collinearity existing between legumes makes possible the transfer of key knowledge from model legumes to faba bean. Here we review the most recent knowledge gained from model legumes on grain quality, resistance to biotic and abiotic stresses, nitrogen-fixing symbiosis and how this knowledge can be employed for faba bean breeding.

Keywords: biotechnology, breeding, faba bean**,** model legumes, *Vicia faba*, *Medicago truncatula*, *Lotus japonicus*

1. Introduction

Grain legumes play a critical role in crop rotation. Thanks to the unique process of biological fixation of atmospheric N_2, grain legumes can meet two major challenges in modern agriculture: (i) reduction of fossil energy use and green house gas emission through decrease of nitrogen fertilizers, which contribute to both CO_2 and N_2O emissions; (ii) diversification of cropping systems to reduce the need for external inputs such as pesticides, to improve nutrient and water use and to reduce losses of nutrients to the environment. However, the inclusion of legumes in the cropping systems is still rather low despite their beneficial functions towards sustainable and multifunctional agroecosystems. To turn grain legumes into proper candidates for a sustainable agriculture, they should be attractive both to producers and to users (human or animal nutrition).

Faba bean is an excellent candidate crop to provide nitrogen input into temperate agricultural systems. Significant genetic variation for symbiotic parameters exists with numerous faba bean germplasm lines maintained, providing an excellent resource for plant breeders (Duc et al., 2009, this issue). Priorities for faba bean breeding are the development of resistant genotypes to biotic (Sillero et al., 2009, this issue) and abiotic constrains such as over-wintering frost (Stoddard et al., 2009a, this issue) and drought (Stoddard et al., 2009b, this issue), and free of anti-nutritional factors (Krepon et al., 2009, this issue). Understanding the bottlenecks during symbiotic signalling and the processes underlying nodule development and nitrogen assimilation are critical for the improvement of current breeding programs. Critical traits to focus on faba bean nodulation biology include high nodulation ability, tolerance to nitrate containing soils, increased symbiotic mass to increase total nitrogen fixation ability, and interaction with mycorrhizal fungi (cf. Meixner et al., 2007). Many of these traits have already been

incorporated into modern cultivars, but several others, many of which are controlled quantitatively by multiple genes, have been more difficult to manipulate. Implementation of Marker-Assisted Selection (MAS) schemes offers plant breeders a mean to improve selection efficiency, reducing the time and effort required to develop new cultivars. Although Quantitative Trait Loci (QTL) mapping studies have been performed for almost all grain legumes, in most cases no markers are readily available for QTL selection and MAS yet. The limited saturation of the genomic regions bearing putative QTLs makes difficult to identify the most tightly-linked markers and to determine the accurate position of QTLs (Torres et al., 2009, this issue). Effectiveness of MAS might soon increase with the adoption of the new improvements in marker technology together with the integration of comparative mapping and functional genomics. Traditional breeding efforts will be greatly enhanced through collaborative approaches using functional, comparative and structural genomics. Development of novel methods to introduce genes into grain legumes through plant transformation methodology promises to give plant breeders the opportunity to overcome hybridization barriers and other limitations related to those traits for which little or no natural resistance has been identified in addition to provide means to study gene function and genome organization. Molecular genetic and genomic analyses promise the transfer of technology from model legumes to faba bean, despite a generation of neglect.

2. The Model Legumes

In the last three decades, the study of complex biological processes in plants has been facilitated by the development of the model plant, *Arabidopsis thaliana*. This model has already allowed various breakthroughs in our understanding of different processes such as plant development (van Hengel et al., 2004; De Smet and Jurgens,

2007) and plant response to biotic and abiotic stresses (Jones and Dangl, 2006; Swindell et al., 2007; Ma et al., 2008). However, while the study and further development of *A. thaliana* as a unique model improved greatly our understanding in some processes, its limits also became apparent. Indeed, *A. thaliana* cannot be considered as the universal model since, for instance, it is not the natural host of many pathogenic or symbiotic bacteria and fungi (Handberg and Stougaard, 1992). Thus, several other species including *Oryza sativa*, *Medicago truncatula* and *Lotus japonicus* have been more recently proposed and developed as alternative models to address specific issues of a more restricted group of plants.

M. truncatula and *L. japonicus* were initially developed as models to study the nitrogen-fixing symbiosis, restricted to the *Fabaceae*, since each species responds slightly differently to its rhizobial partner: *L. japonicus* forms determinate nodules whereas *M. truncatula* forms indeterminate nodules (Handberg and Stougaard, 1992; Rose, 2008). The fact that both are small self-fertile plants with a short growth cycle and profuse flowering and seed production makes them ideal for classical and molecular genetics since fast generation of a mapping population is easy to achieve. In addition, *M. truncatula* and *L. japonicus* are diploid legume species with eight and six chromosomes in their haploid phase respectively (Barker et al., 1990; Handberg and Stougaard, 1992). Their genomes are relatively small estimated around 500 and 470 Mb respectively (Sato et al., 2007), so only slightly higher than *A. thaliana* (125 Mb) and significantly smaller than most legume species i.e. 4,000 Mb for pea or 13,000 Mb for faba bean (Barker et al., 1990; Handberg and Stougaard, 1992). Altogether, these characteristics, along with the capabilities of these species to be transformed by *Agrobacterium tumefaciens* or *A. rhizogenes,* have made of *M. truncatula* and *L.*

japonicus valuable tools for the dissection of symbiotic interactions at the molecular level and to address specific legume needs.

To facilitate the study of plant-microbe symbiotic interaction in legumes, different tools for classical, molecular and reverse genetics, along with functional genomics were developed in these two species. Several germplasm collections of both model species are available that are useful to search for genetic polymorphism for particular traits. Mining these collections allowed the development of several genetic maps, based on F2 populations, in both species using a wide array of genetic markers such as Cleaved Amplified Polymorphic Sequence (CAPS), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD) and microsatellites that are consolidated by the generation of Recombinant Inbred Lines (RILs) (Thoquet et al., 2002; Choi et al., 2004a; Sandal et al., 2006; Wang et al., 2008). Among these maps, the intra-specific maps in *M. truncatula* A17 x A20 (Ané et al., 2008) and in *L. japonicus,* Gifu B129 x Mijakojima MG-20 (Wang et al., 2008), and the inter-specific map between *L. japonicus* Gifu B129 and *L. filicaulis* (Sandal et al., 2006) have been used as reference for map-based cloning and genome sequencing. The information obtained by these genetic maps was complemented by the generation of cytogenetic maps based on Fluorescence *in-situ* Hybridisation (FISH) on pachytene chromosomes, instrumental for map-based cloning and comparative genomics (Kulikova et al., 2001; Pedrosa et al., 2002). In addition, a genome sequencing initiative of gene-rich regions *via* the Bacterial Artificial Chromosome (BAC)-by-BAC strategy and different Expressed Sequence Tag (EST), sequencing programs have been initiated for *M. truncatula* and *L. japonicus*. These programs have led to the creation of more than 200,000 and 100,000 ESTs, available from public DNA database, for *M. truncatula* and *L. japonicus*, respectively,

and the sequencing of nearly 190 and 315.1 Mb of their respective genomes (Cannon et al., 2006; Ané et al., 2008; Sato et al., 2008).

In parallel, transcriptomic and proteomic tools have also been developed in both models as alternative approaches for the study of the symbiotic interaction and other processes (Wienkoop and Saalbach, 2003; Colebatch et al., 2004; El Yahyaoui et al., 2004; Kouchi et al., 2004; Hohnjec et al., 2005; Gallardo et al., 2007; Sanchez et al., 2008). Several macro- and micro-array platforms have been developed in these species initially to study the symbiotic interaction. Large-scale macro-array techniques allowed the monitoring of 6,000 and 15,000 genes simultaneously in *M. truncatula* and *L. japonicus* (El Yahyaoui et al., 2004; Kouchi et al., 2004). Targeted macro-array platforms were also developed to study specific topic including a 92 defence-related gene macro-array to study the *M. truncatula-Colletotrichum trifolii* interaction (Torregrosa et al., 2004) or a 384 salt stress-related genes to study *M. truncatula* response to salt stress (Merchan et al., 2007). Simultaneously, the first micro-array platforms, developed at the University of Bielefeld (Germany) and the Max Planck Institute of Molecular Plant Physiology, Golm (Germany) allowed the screening of 6,231 and 2,500 unique transcripts respectively (Colebatch et al., 2004; Küster et al., 2004). With the progress of genome sequencing, the *M. truncatula* micro-array platform was upgraded to allow the monitoring of 16,000 genes (Hohnjec et al., 2005) that have also been completed with the entire *Sinorhizobium meliloti* genome to develop a dual symbiotic chip (Barnett et al., 2004). In addition, Affymetrix chips with bioinformatically optimized oligonucleotides are also commercially available for *M. truncatula* and *L. japonicus* (http://www.affymetrix.com; Sanchez et al., 2008) and a novel generation of *M. truncatula* gene chips with probe sets for 1,850 *M. sativa* transcripts to facilitate transcriptomic analysis of closely related species will be soon

available (Ané et al., 2008). In parallel to these large-scale hybridisation techniques, a method for the simultaneous monitoring of more than 700 transcription factors by quantitative Polymerase Chain Reaction (PCR) has been established at the Max Planck Institute of Molecular Plant Physiology, Golm (Germany) (Kakar et al., 2008). All these transcriptomic platforms allowed large improvements in our understanding of legume symbiotic interactions and begin to be used for other purposes including the response to salinity (Sanchez et al., 2008; Gruber et al., 2009), grain filling (Gallardo et al., 2007; Verdier et al., 2008), or legume-pathogen interactions (Torregrosa et al., 2004; Curto et al., 2007; Ameline-Torregrosa et al., 2008; Dita et al., 2009).

In turn, several proteomic approaches, based on different protein separation methods and identification by mass spectrometry, have been developed and applied for these model species. These original approaches targeted the establishment of reference protein and peptide maps in *M. truncatula* (Watson et al., 2003) and the analysis of the symbiotic compartment in both *L. japonicus* and *M. truncatula* (Wienkoop and Saalbach, 2003; Valot et al., 2006; Larrainzar et al., 2007; van Noorden et al., 2007). More recently, the range of application of proteomic approaches has been broadened to include grain filling (Gallardo et al., 2003; Gallardo et al., 2007; Repetto et al., 2008) and pathogen interactions (Colditz et al., 2005; Castillejo et al., 2009). Nowadays, second and third generation proteomic tools such as Differential In-Gel Electrophoresis (DIGE) and isobaric Tag for Relative and Absolute Quantitation (iTRAQ) are being developed in *M. truncatula* along with approaches targeting the post-translational modifications including nitrosylation and phosphorylation at large scale (M.A. Castillejo, personal communication).

Apart from these genomic tools, many reverse genetic approaches were also developed in these models. To this purpose, several collections of chemical or

insertional mutants including T-DNA and transposon tagged lines have been created (Thykjaer et al., 1995; Penmetsa and Cook, 2000; Webb et al., 2000; Kawaguchi et al., 2002; Tadege et al., 2008). These collections have already been used to identify new genes required for symbiosis but may also be screened for other interesting traits. The improvement of PCR-based techniques for screening for mutation in gene of interest allowed the development of novel approaches for efficient reverse genetic analysis. Several of these novel approaches, have been or are being developed in *M. truncatula* and *L. japonicus* including Targeted Induced Local Lesions in Genome (TILLING), saturating *Tnt*1-insertion mutagenesis and fast-neutron mutagenesis. TILLING relies on point mutagenesis with ethyl methyl sulfonate (EMS), and provides an allelic series ranging from silent mutations to complete loss-of-function of the gene of interest. This method was first developed for legume in *L. japonicus* (Perry et al., 2003) already allowing the identification of novel symbiotic genes in this species (Heckmann et al., 2006; Horst et al., 2007) and is now available in *M. truncatula* (the Grain Legumes European Integrated Project (GLIP), http://www.eugrainlegumes.org/). In an attempt to saturate the whole *M. truncatula* genome, a large collection of *Tnt1*-tagged *M. truncatula* lines has been established along with the facilities to characterise the transposon-flanking regions (Tadege et al., 2008). Fast-neutron mutagenesis detection methods were also set-up for *M. truncatula* and *L. japonicus,* allowing the identification of one non-nodulating mutant FNN5.2 in *L. japonicus* (GLIP, http://www.eugrainlegumes.org/; Hoffmann et al., 2007). In addition to help identifying new genes involved in plant biology, these methods can serve to identify the exact function of these genes, which is a pre-requisite step before gene transfer into other legume crops such as faba bean. *Tnt1* mutagenesis and related transposon or T-DNA tagging which insert within the gene and Fast neutron bombardment, which generates

large deletions, are likely to produce gene knockouts, ideal to identify gene function but the point mutants identified by TILLING may be more useful as a source of favourable alleles for subsequent selection. Alternatively to these mutation-based methods, two transformation-based methods, RNA interference (RNAi) and/or Virus-Induced Gene Silencing (VIGS) were also established in these model legumes (Limpens et al., 2004; Maeda et al., 2006).

Altogether all the resources developed in these two models make them ideal candidates to study legume physiology and have already provided important breakthroughs in our understanding of legume symbiosis. In addition, these two species are also affected by most stresses limiting legume crop yield such as fungal and bacterial diseases, nematodes, pests or salt stress so that the different resources developed on these species provide a great advantage to improve our understanding and the breeding for the specific needs of legume crops such as faba bean.

Alternatively of these two model legumes, several legume crops including soybean and pea, have been extensively studied due to their economical importance. These two crop species count on large collections of germplasms and chemical and insertional mutants and are the subject of genomic sequencing initiatives. In addition, many genomic tools have been and are being developed in these species including proteomic and transcriptomic platforms and functional genomic approaches such as TILLING, RNAi and VIGS silencing techniques (Constantin et al., 2004; Subramanian et al., 2005; Zhang and Ghabrial, 2006; Cooper et al., 2008; Dalmais et al., 2008; Kaimoyo and VanEtten, 2008). Thus these two legume crops can also serve as model to transfer interesting traits to faba bean.

3. Synteny Between *M. truncatula, L. japonicus* and Grain Legumes

Depending on the degree of their evolutionary relationship, different species preserve similarities in the content, proximity (synteny) and linear order (collinearity) of genes in their genomes. This suggestion derives from the idea that the relative location of genes in a genome is an accident of history and that the decay of colinearity is simply a function of chance and divergence time. Comparative mapping and genome analysis investigate conservation and differences in gene content and order among different taxa. Originally comparative analyses of genomes were performed based on genetic maps developed with molecular markers, but the increasing availability of large-scale genome sequences (www.plantgdb.org/prj/Genome_browser.php) could make comparison more direct and extensive. In the last three decades, multiple studies, using linkage maps, revealed remarkable synteny predominantly within plant families (Paterson et al., 2000). Conserved gene order between species from distinct plant families was also identified within small-scale genomic regions (microsynteny) rather than simply among large chromosome segments (Devos et al., 1999; Stracke et al., 2004; Kevei et al., 2005; Zhu et al., 2005 and many others), although collinearity between large chromosomal blocks of markers across species separated by wider taxonomic distances has also been identified in a few cases (Lee et al., 2001; Wu et al., 2006).

Comparative genetic analysis among legumes species was launched by Vavilov's studies (1922) on series of similar heritable variations in related Papilionoid species. The first molecular proofs for the existence of macrosynteny between legumes were given by the comparison of genetic maps of economically important grain legumes. The comparison of the incomplete genetic maps of lentil (*Lens culinaris*; 2n=14) and chickpea (*Cicer arietinum*; 2n=16) with the pea linkage map revealed eight and five large syntenic blocks respectively (Weeden et al., 1992; Simon and Muehlbauer, 1997;

Ellis and Poyser, 2002). Comparison between pea and *M. sativa,* also revealed a substantial conservation in the gene order in these species. This comparison allows to identify the genetic rearrangements that occurred and account for their chromosome number difference (8 for alfalfa and 7 for pea; Kaló et al., 2004), which also indicated that the 10-fold difference in their genome size is not the result of large scale pea genome multiplication. Completing the *M. truncatula* genetic map with *M. sativa* gene-based genetic markers (Kaló et al., 2000) identified the homologous linkage groups and showed an almost complete colinearity between these two related species except for the rDNA chromosomal localization (Choi et al., 2004a). For the Phaseolid legumes, a high level of collinearity and synteny was detected as shown between the genome of several *Vigna* species (Menancio-hautea et al., 1993; Kaga et al., 2000) and between *V. radiata* (mungbean) and the phaseolid legumes *Dolichos lablab* (Humphry et al., 2002) and common bean (Boutin et al., 1995). Comparison of genetic maps between soybean and common bean revealed only short conserved linkage blocks in common bean that often corresponded to nearly entire linkage groups or large contiguous blocks in soybean (Boutin et al., 1995; Lee et al., 2001).

The genomic resources developed for *M. truncatula*, *L. japonicus,* soybean and common bean boosted comparative genomic analyses between model legumes and legume crops by allowing more comprehensive macrosynteny analyses such as those reported by Choi et al. (2004b) and Zhu et al. (2005). Cross-species gene specific markers were used to identify homologous genome segments among eight legume species (*M. truncatula*, alfalfa, *L. japonicus*, pea, chickpea, soybean, mungbean and common bean). Using the *M. truncatula* genetic map as a reference genome, the eight legume genomes were aligned and a simplified consensus map was created. The degree of collinearity between legumes reflected their phylogenetic relationship. The large

amount of genomic sequences generated between the two model legumes *M. truncatula* and *L. japonicus* allows a more comprehensive in-clade comparison showing several macrosyntenic regions and significant microsyntenic regions conserving many genes in the same order and orientation (Fig. 1; Choi et al., 2004b; Cannon et al., 2006). The comparison of the microstructure of the *MtDMI2*(*NORK*)/*LjSYMRK* region also revealed a nearly complete conservation in gene content and order between the two species within a 276 kb long *M. truncatula* chromosomal segment (Kevei et al., 2005; Zhu et al., 2005). The comparative mapping between *M. truncatula* and soybean identified eleven colinear blocks with a high degree of microsynteny (Choi et al., 2004b; Mudge et al., 2005). Similarly, segments of eight linkage groups of common bean (2n=22) exhibited conservation with *M. truncatula* linkage groups (Choi et al., 2004a; 2004b).

A similar approach – generating intron-targeted gene-based anchor markers for legume species and using *M. truncatula* as a reference genome for comparative mapping - was applied in the comparative mapping program of GLIP (http://www.eugrainlegumes.org/) to analyse macrosyntenic relationship between pea, chickpea, faba bean, common bean, lupin and lentil. The alignment of the legume genetic maps is currently underway and preliminary data show that high level of macrosynteny exists between the genomes of *M. truncatula*, lentil (Phan et al., 2006), faba bean and chickpea (Gutierrez et al., 2008b; P. Winter personal communication). These analyses also indicated a complex syntenic pattern between *M. truncatula* and lupin for which individual *M. truncatula* chromosomes were syntenic to at least two lupin linkage groups accounting to the higher lupin chromosome number (Nelson et al., 2006; Phan et al., 2006; Phan et al., 2007). In parallel, syntenic analysis between *M.*

truncatula, *L. japonicus* and peanut (*Arachis hypogea*), a more distant grain legume, revealed significant macrosynteny between these species (Hougaard et al., 2008).

These recent comparative genomic studies have mainly used *M. truncatula* as a reference genome and revealed that colinearity exits between legume species to different extents depending on their phylogenetic distance. In order to support comparative legume biology the Legume Information System (LIS) was developed (Gonzales et al., 2005) few years ago, which integrates genetic and physical map data and enables macrosynteny analyses to be carried out between legume species *in silico*.

Both *M. truncatula* and faba bean are cool season legumes falling into two separate tribes; faba bean (2n=12) belongs to the *Viciae* and *M. truncatula* is a species in *Trifoliae* tribe (Zhu et al., 2005). The genome size of faba bean is about 25-fold larger than the genome of *M. truncatula,* which restrained the development of faba bean genomics. A composite genetic map of *V. faba* has been constructed (Román et al., 2004) and, in the frame of GLIP, intron-targeted gene-based markers have been developed and tested for faba bean. The comparative mapping between faba bean and *M. truncatula* is in progress (Gutierrez et al., 2008a). Based on the phylogenetic distance between the two species, large scale genome conservation is expected and the identification of chromosome rearrangements responsible for different chromosome number is likely, as it has been detected between *M. truncatula* and pea (Choi et al., 2004b; Kaló et al., 2004). The expected high level of conservation in gene order between faba bean and *M. truncatula* implies that *M. truncatula* genomic tools will facilitate breeding and research of faba bean.

4. Strategy for Faba Bean Improvement

A major aim for any crop breeding program is the development of good quality lines with an adequate resistance/tolerance to yield-reducing stresses. The use of model legumes for comparative functional genomics may bring some new perspectives and enhance faba bean breeding efforts. In this way, identification of QTLs and/or candidate genes involved in stress tolerance and/or quality may be used to produce transgenic lines and/or these traits can be applied to breeding programs (e.g., MAS).

Little is known about the functional correspondence of model legume genes and their putative faba bean orthologues. Notwithstanding the lack of information, predictions can be made based on the sequence similarities between the relatively few *M. truncatula* and faba bean gene pairs that are available and the high conservation and synteny existing between legume genomes. Whereas for highly conserved genes, favourable mutations observed in model legumes are likely to correspond to favourable alleles in faba bean, for less conserved genes (i.e. many transcription factors), the relation is less reliable. Possible complications include 1) differences in gene copy number, 2) differences in transcript or protein abundance, 3) differences in specific activity. Therefore, the information obtained in model legumes can be used as a guide to narrow down candidate genes, but proof can only come from functional studies, preferably in the homologous system.

Once a series of candidate genes to improve a particular trait has been identified in one of the model legumes, a number of options are possible for exploiting this information in legume crops and particularly in faba bean breeding. The involved steps are: 1) confirmation of candidate gene function either directly in faba bean or indirectly in any of the model legumes, 2) identification of favourable alleles for selection, 3) variety improvement by MAS or by transformation of an elite line.

Several approaches have been developed to confirm candidate gene function at the biochemical and physiological level. Originally, functional analysis of proteins was performed through two main techniques, protein over-expression and monitoring of promoter activity. Over-expression of a candidate gene is obtained by transferring the coding region of the gene under control of a strong promoter such as the *CaMV 35S* into the plant and function is assigned by scoring the phenotype of the resulting transformed line (Shimoda et al., 2008; Vernié et al., 2008). Promoter activity analysis is performed by linking the promoter sequence to reporter gene such as the beta-glucuronidase (GUS) or the green fluorescent portein (GFP) to allow analysis of tissue-specific expression (Hayashi et al., 2008). Both procedures require gene transfer that is difficult in large seeded legumes. This limitation can often be short-cut by hairy root transformation that is easier to achieve but only allows analysis of gene constructs in root tissue. Albeit with low efficiency, protocols for both *A. tumefaciens* and *A. rhizogenes* transformation have been established for faba bean and can be used for gene functional analysis in this species (Böttinger et al., 2001; Vieweg et al., 2004). Alternatively, the functional analysis could be performed in the model legumes *M. truncatula, L. japonicus* or soybean for which the transformation protocols are more efficient and rapid (Lombari et al., 2003; Crane et al., 2006; Kereszt et al., 2007; Rech et al., 2008).

In these model legumes, gene function can also be removed by modern molecular genetic techniques including RNAi (Wesley et al., 2001), VIGS (e.g. Kachroo et al., 2008) and even TILLING (Colbert et al., 2001). The TILLING approach is also available for *G. max* (Cooper et al., 2008) and *P. sativum* (Dalmais et al., 2008) but not yet for faba bean, the difficulty being generation and maintenance of a large perfectly homozygous population for mutagenesis.

Once the function of a candidate gene has been validated, identification of favourable alleles has to be performed. Defining patterns of synteny and collinearity between species by comparative genomic studies (cf. section 3) helps the identification of orthologous genes in genetically recalcitrant species as compared to model systems. Once a gene behind a given phenotype has been identified by a map-based cloning approach and validated, the orthologous gene in the other species can be isolated based on similar map position. There are several examples for this fruitful approach among the legume species where genes involved in symbiotic interactions have been identified (e.g. pea mutants *sym19* - *DMI2/NORK*, *sym2* - *LYK*3, *sym7* - *NSP2*; Endre et al., 2002; Limpens et al., 2003; Kaló et al., 2005). The syntenic map position of the dwarf phenotype in diploid alfalfa (*Msdwf1*) and pea (*le*) and the genomic resources in *M. truncatula* enabled the identification of a gene encoding a gibberellin 3-β-hydroxylase (GA3ox) required for normal growth habit in diploid alfalfa (Dalmadi et al., 2008). These examples clearly demonstrate the two-way utility and application of molecular markers and the identified orthologous regions between the genomes of reference and crop legumes. The tools developed in model species can facilitate the identification of agronomically important genes (QTLs, genes involved in nutrient quality and quantity, biotic and abiotic stresses, etc.) and marker-assisted breeding programs in target organisms while the accumulated biological knowledge in crop species can contribute the understanding of biological processes. Alternatively, selection of favourable alleles of the gene of interest can be found using the EcoTILLING approach that allows the detection of allelic variants of a candidate gene in natural populations for their subsequent phenotyping for the trait in question. Finally, the favourable allele can be transferred to elite faba bean cultivars by MAS or genetic transformation.

5. Application of Model Legumes to Faba Bean Improvement

5.1. Breeding for quality

5.1.1. Model legumes and quality traits

M. truncatula seed biology is essentially very similar to that of the major temperate crop legumes, pea and faba bean, but differs in that the major carbon reserves are lipids, rather than starch, which is present only in trace amounts in the mature seed. The *M. truncatula* seed also contains about 10% of endosperm material at maturity, unlike pea or faba bean in which this layer is reabsorbed during development. As *M. truncatula* was not bred for grain consumption, its seeds are also relatively small with a relatively high proportion of cell wall material and a low harvest index. Proteins represent the major class of storage compounds in *M. truncatula* seeds, followed by lipids, with only trace quantities of starch (Duc, 2004; Djemel et al., 2005). Whereas proteins and oils are coordinately synthesized during seed filling, the non-starch carbohydrate fraction (mainly trachyose) accumulates only at the end of seed maturation, when seeds are acquiring desiccation tolerance. Fatty acid and sugar compositions are similar to those of pea and other grain legumes. Thus, with certain caveats, the *M. truncatula* seed is a good model for identifying genes important in regulating seed composition in grain legumes.

5.1.2. Identification of grain quality characters

The availability of a comprehensive EST database has allowed a large-scale identification of genes putatively encoding *M. truncatula* seed proteins that have been subsequently confirmed by seed protein separation and Matrix-Assisted Laser Desorption/Ionization – Time-of-Flight (MALDI-TOF) analysis, some of which are candidate genes for quality traits (Watson et al., 2003; Gallardo et al., 2003). The major

M. truncatula storage proteins are the 7S (vicilin and convicilin-type) and 11S (legumin-type) globulins, with similar amino acid compositions to those of other grain legumes, notably being poor in sulphur-containing amino acids (Gallardo et al., 2003). The storage proteins accumulate sequentially during seed filling, the vicilins at 14 days after pollination (DAP) followed by the legumins (16 DAP) with the convicilins accumulating last (18 DAP).

Among the proteins identified at different developmental stages, several enzymes and other proteins playing key roles in the seed were detected. For example, cell division-associated proteins were expressed during the differentiation phase preceding seed filling. Storage protein accumulation was accompanied by the expression of putative chaperonins and protein disulphide isomerases. During this phase, two PV100-like polypeptides also accumulate (Yamada et al., 1999) giving rise to a trypsin inhibitor and a cytotoxin-related peptide upon processing, which are important targets for breeding as their elimination could improve nutritional quality of legume seeds.

Starch accumulates only transiently in *M. truncatula* seeds, in contrast to the starch-rich pulse pea and faba bean, but similarly to the situation in soybean, starch remobilisation is the contributing carbon source for oil biosynthesis (Duc, 2004). Certain starch-remobilisation enzymes (starch synthase, sucrose synthase and triose phosphate isomerase) were transiently expressed 16-24 DAP, concomitantly with proteins involved in photosynthesis, supporting the hypothesis that photosynthesis in the embryo provides energy for lipid biosynthesis, which may also recycle fixed CO_2 (Gallardo et al., 2003).

Seed development involves the interplay of several tissues; the developing embryo is surrounded by the endosperm, and the two organs are embedded in the

maternal integument. The role played by the embryo-surrounding tissues in legume seed reserve accumulation has been investigated genetically for pea and soybean (Lemontey et al., 2000), indicating significant maternal effect early in seed filling. To study these interactions in more detail and get access to the genes involved, gene expression in these tissues has been analysed at the proteome and transcriptome levels (Gallardo et al., 2007). A general observation is that the pattern of proteins and transcripts expressed in the embryo, endosperm and integument is very specific for each cell type, with little overlap. One of the major findings was an extensive compartmentalization of amino acid metabolism between seed tissue components that may favour storage product accumulation. Of particular interest is the compartmentalization of enzymes of sulphur amino acid biosynthesis, observed for both methionine and cysteine, as these are limiting in grain legumes.

The dependence of the embryo's nutrition on the maternal tissue was also demonstrated directly by an *in vitro* culture experiment in which embryo development on nitrogen nutrient-free medium with and without the surrounding tissue was compared (Gallardo et al., 2006). Embryos grown without nitrogen source aborted, whereas embryos grown in presence of the surrounding endosperm and integument developed normally and accumulated reserve proteins, presumably due to nitrogen remobilisation from maternal tissues. This remobilisation of a temporary nitrogen store requires proteolysis, and candidate proteases with appropriate expression kinetics have been identified in endosperm and seed coat tissues (Gallardo et al., 2007).

Seed developmental programme is under tight transcriptional control, and there is evidence from other plant systems that an important class of loci regulating seed composition corresponds to transcription factors (Le et al., 2007). To identify transcription factors (TFs) expressed in developing *M. truncatula* seeds, expression of

more than 700 TF sequences was monitored by quantitative real-time PCR throughout seed development (Verdier et al., 2008). By clustering the data of TF expression with storage protein expression profiles previously obtained, candidate factors potentially controlling the major storage protein groups were identified. In parallel, a biochemical approach analysing the nuclear proteome led to the identification of several putative regulatory proteins (Repetto et al., 2008), the functions of which remain to be determined by reverse genetics. Identified genes and proteins from all these studies may serve as quality markers potentially transferable to crop legumes for breeding once their involvement in seed quality is determined and polymorphism for these traits are found.

A survey of natural variations in seed protein complements carried out on 50 diverse *Medicago truncatula* ecotypes or cultivars indicated a high degree of polymorphism in protein composition and a large variation in protein content (33-46%) (Le Signor et al., 2005). Clustering of genotypes according to similarity in one-dimensional protein profiles allowed structuring into classes that corresponded to 4 species groups within the *M. truncatula* species complex. This classification has allowed the selection of RIL parents for maximizing variation in protein content and type in the populations to be examined, and mapping of QTLs is in progress. In a new project, expressional gene candidates, selected from the cited studies, are being mapped directly on the genetical-physical *M. truncatula* map for comparison with the positions of mapped traits. So far, around 50% of the gene candidates have been mapped, giving a total of around 750 loci, including transcription factors, nutrient transporters and other seed-specific enzymes of metabolism (A. Bordat, personal communication). A survey of QTLs for traits affecting vegetative plant development and seed yield and content in pea (Burstin et al., 2007) revealed the importance of genes determining plant architecture in controlling seed yield and protein content. It would appear likely that the homologous

loci in faba bean have the same properties, and therefore these should form part of selection schemes.

Apart from selection for seed size and seed number per pod, quality breeding in faba bean has to date concentrated on the reduction/elimination of the anti-nutritional factors condensed tannins, vicine and convicine, responsible for favism, a severe digestive disorder in susceptible individuals, which reduce nutritional value of faba bean (Gutierrez et al., 2006; Gutierrez et al., 2007; Gutierrez et al., 2008a). CAP markers have been obtained for the convicine locus *v-c*, and SCAR markers for the tannin loci *zt-1* and *zt-2* for use in introgression of favourable alleles in breeding selection. Linked molecular markers such as these may be subject to recombination with the trait of interest. With the sequence data available from *M. truncatula*, there should be the possibility of identifying genes encoding the responsible enzymes, and thus of obtaining non-recombining and hence more reliable SNP markers within the gene itself. The development of inbred lines, perhaps based on the closed flower mutation (Poulsen, 1977), would facilitate genetic analyses.

5.2 Breeding for resistance to biotic stresses

Grain legume and in particular faba bean are challenged by many pathogens and pest including bacterial, virus and fungal diseases as well as infection by nematodes and some parasitic plants which strongly affect crop yield worldwide (see Pérez-de-Luque et al., 2009, this issue; Sillero et al., 2009, this issue; Stoddard et al., 2009c, this issue). Genetic resistance is considered the most desirable control method since it is more cost effective and environment-friendly than the use of chemicals. Thus, many resistance sources (Sillero et al., 2009, this issue) and their associated QTLs have been found in different grain legumes including faba bean (Torres et al., 2009, this issue). However,

the long genetic distance existing in most cases between the identified genetic markers and the resistance QTLs, the common lack of codominant markers and the general lack of knowledge on resistance mechanisms in legumes limit greatly the use of genetic markers to confer resistance to grain legumes. The model legumes *M. truncatula* and *L. japonicus* are affected by many of the pathogens and pest limiting faba bean yield. Thus, they offer a great opportunity to improve the knowledge in resistance mechanisms against faba bean pathogens and identify effective resistance genes against them.

Fungal and oomycete pathogens are the most diverse group of pathogens and cause the most dramatic damages on legume yield worldwide. Annual *Medicago* and *M. truncatula* in particular are strongly affected by a wide range of foliar and soil-borne necrotrophic fungi which makes of *M. truncatula* a promising model to study the plant-necrotrophic fungi interaction (reviewed in Tivoli et al., 2006). Several studies revealed that *M. truncatula* is a potential host not only of necrotrophic fungi but also of several biotrophic fungal and oomycete pathogens including *Aphanomyces euteiches* (Moussart et al., 2007), *Colletotrichum trifolii* (O'Neill and Bauchan, 2000), *Erysiphe pisi* (Prats et al., 2007), *Fusarium* spp. (Barbetti and Allen, 2005), *Leptosphaerulina trifolii* (Barbetti, 2007), *Mycosphaerella pinodes* (Moussart et al., 2007), *Phoma medicaginis* (O'Neill et al., 2003; Ellwood et al., 2006; Barbetti, 2007), *Peronospora trifoliorum* (Yaege and Stuteville, 2000), *Uromyces striatus* (Rubiales and Moral, 2004). In most cases, screening of germplasm collections of *M. truncatula* allowed identification of a wide range of differential responses to the pathogen from highly susceptible to resistant (Moussart et al., 2007; Prats et al., 2007). This serves as bases for the characterisation of underlying resistance mechanisms at the cellular and molecular levels as well as for the identification of defence genes and QTLs responsible for resistance.

M. truncatula resistance against *P. medicaginis* and *C. trifolii* was found to be controlled by single major genes, named *rnpm1* and *RCT1* respectively. These major genes localised at the top of the linkage group 4 in a region containing a cluster of several nucleotide binding site (NBS) – leucine rich repeat (LRR) proteins that are often plant resistance (R) genes (Torregrosa et al., 2004; Yang et al., 2007; Kamphuis et al., 2008). Interestingly, the RCT1 gene of *M. truncatula* has been successfully transferred to alfalfa to confer anthracnose resistance (Yang et al., 2008). Resistance to *A. euteiches*, *M. pinodes*, *U. striatus*, *P. trifoliorum* or *E. pisi* appears to be controlled by different defence mechanisms. For instance, screening of an USDA collection of *M. truncatula* germplasms for *E. pisi* resistance indicated that resistance to powdery mildew was controlled by papilla formation, by early hypersensitive response and also by post-haustorial mechanisms (Prats et al., 2007). Mapping of the QTLs controlling resistance to these fungal pathogens in *M. truncatula* is now underway (D. Rubiales, personal communication).

In parallel, the transcriptomic and proteomic approaches developed for this model legume are being used to understand the molecular components and to identify candidate genes involved in *M. truncatula* defence against these fungal pathogens. For instance, a Substractive Suppression Hybridisation (SSH) library indicated that Pathogen-Related (PR)10 proteins and proteins associated with abscisic acid signalling play important roles in the *M. truncatula* resistance against *A. euteiches* (Nyamsuren et al., 2003). The crucial role of PR10 in *A. euteiches* resistance was confirmed by comparative proteomic and gene silencing approaches, which indicated that PR10 silencing led to increased resistance by antagonist induction of other PR genes (Colditz et al., 2004; Colditz et al., 2007). Comparison of the proteomic profile of several *M. truncatula* lines with varying levels of resistance also identified other proteins

potentially involved in *A. euteiches* resistance such as proteasome alpha subunits (Colditz et al., 2005). Comparison of expression profiles of 92 defence-related genes by macroarray between a resistant and a susceptible line of *M. truncatula* at key steps of *C. trifolii* infection also highlighted the important role of PR proteins and in particular PR10 in resistance. As expected, this analysis indicated that a large proportion of genes present on the macroarray membrane were upregulated in the resistant *M. truncatula* line while these genes were mainly downregulated in the susceptible line. Microarray analysis of several *M. truncatula* genotypes with different defence mechanisms against *E. pisi* allowed the identification of a set of genes involved in these defence mechanisms (Curto et al., 2007; Foster-Hartnett et al., 2007). Post-genomic approaches are also being applied to tackle other fungal diseases such as *M. pinodes* (Fondevilla et al., 2008) and *U. striatus* (Madrid et al., 2008).

Legumes are also affected by bacterial pathogens. In particular, *M. truncatula* can be infected by the causing agent of the bacterial wilt disease, *Ralstonia solanacearum,* which also infects a large number of crops including tomato, potato and cultivated legumes such as faba bean. A recent study showed that one *M. truncatula* line, F83005.5, susceptible to *C. trifoliorum* and *P. medicaginis*, was resistant to most *R. solanacearum* isolates (Vailleau et al., 2007). A major QTL was mapped on chromosome 5 and two minor ones on chromosome 3 and 7 that may be helpful for MAS (Vailleau et al., 2007).

Nematodes are also an important cause of yield losses in legumes. Interestingly, *M. truncatula* and *L. japonicus* have been shown susceptible to most nematodes affecting legumes. For instance, *M. truncatula* can be colonised by the stem nematode *Ditylenchus dipsaci*, causing disease in many legumes such as alfalfa, pea and faba bean (Plowright et al., 2002; Moussart et al., 2007). By screening a *M. truncatula* germplasm

collection, Moussart et al. (2007) identified several resistant and susceptible *M. truncatula* lines that will surely allow a better understanding of stem nematode-legume interaction. *L. japonicus* and *M. truncatula* are also infected by different root-knot and cyst nematodes belonging to the *Meloidogyne* and *Heterodera* genera. Interestingly, Weerasinghe et al. (2005) showed, in *L. japonicus,* that root-knot nematode and rhizobium interactions may share common pathways. Indeed they found that *L. japonicus* mutants deficient for nitrogen-fixing symbiosis establishment were more resistant to *Meloidogyne incognita* than the wild type while a hypernodulating mutant was infected to a higher extent by the nematode (Weerasinghe et al., 2005). On the other hand, screening of *L. japonicus* ecotypes revealed differential infection responses according to the ecotype ranging from susceptible to resistant to this nematode. Such genetic diversity is being used to map and identify genes and/or QTLs involved in root-knot nematode resistance (Poch et al., 2007).

Although less studied, legumes are also under the thread of viruses. Despite the damage they cause, very little is known about virus resistance mechanisms and nearly no studies have aimed at the characterisation of virus resistance in the two model legumes *M. truncatula* and *L. japonicus*. The only report published to date indicated that *L. japonicus* could be infected by *Arabis mosaic virus* and *Tobacco ringspot virus* while it was resistant to most legume infecting viruses (Schumpp et al., 2007). Due to their economic importance, virus diseases have been more studied in soybean, which thanks to its relatively small genome and the development of genomic tools begins to be considered as the third model legume (Maroof et al., 2008b). In this species, several resistance genes to the soybean mosaic virus have been identified and pyramidized in a single cultivar (Maroof et al., 2008a). In parallel, Babu et al. (2008) found that during the susceptible interaction, the defence reaction was only activated at the latest stages of

the interaction, which may be critical for the systemic infection of the virus. Independently, several transgenic approaches have been undertaken leading to increased resistance against several viruses including the soybean mosaic virus (Furutani et al., 2007) and the soybean dwarf virus (Tougou et al., 2007).

In semi-arid regions worldwide, including Southern and Eastern Europe, North and East Africa and the Middle East, parasitic plants of the *Orobanche* spp. including *O. crenata*, *O. aegyptiaca* and *O. foetida* drastically decrease legume yield. *M. truncatula* has been recently proposed as a model to study the interaction *Orobanche* spp. – legumes (Rodriguez-Conde et al., 2004; Lozano-Baena et al., 2007; Fernández-Aparicio et al., 2008). *L. japonicus* can be infected by *O. aegyptiaca*, but shows incompatible interaction against *O. minor*, *Striga hermonthica* or *S. gesnerioides* (Kubo et al., 2009). Even when *L. japonicus* is not infected by *O. minor*, its root exudates have strong stimulatory activity of *O. minor*, as well as of *O. crenata*, *O. densiflora*, *O. aegyptiaca* and *O. ramosa* seeds (Fernández-Aparicio et al., 2009).

To improve our understanding of the *M. truncatula*-*O. crenata* interaction, a SSH library has been created, allowing the identification of around 300 candidate genes for *O. crenata* defence (Dié et al., 2007). In addition, a microarray analysis of the *M. truncatula* genes regulated in response to *O. crenata* was recently performed on the M16kOLI1 microarray platform (M.A. Dita, unpublished). A comparison of two-dimension proteomic profile of two *M. truncatula* genotypes varying in their level of resistance against *O. crenata* was also performed (Castillejo et al., 2008). Preliminary analysis of the comparison of the transcriptome of two *M. truncatula* genotypes with different resistance mechanisms indicated significant changes in the steady-state level of many transcripts belonging to several functional categories, including pathogen-induced genes, such as PR genes, hormone-associated genes and transcription factors. These

analyses also revealed the activation of both the salicylic acid and jasmonate defence-pathways (M.A. Dita, unpublished). These preliminary results support the previously established results and should prove useful to identify potential candidate genes for crop improvement. These candidate genes should be validated through functional analysis. Validated candidates may then be used for genetic improvement of crop either directly through genetic transformation or indirectly by MAS.

5.3. Breeding for resistance to abiotic stresses

Global climate change predictions suggest new scenarios with larger arid areas and extreme climatologic events. Thus, it is essential to understand how plants respond to different abiotic stresses in order to improve crop performance. This difficult task can only be achieved by integrating conventional breeding and biotechnological approaches (Chaves et al., 2003). However, most legume crops are not easily amenable for molecular and genetic studies. To circumvent this limitation knowledge gained on the two model legumes *M. truncatula* and *L. japonicus* may be further used to understand the responses to abiotic stresses in other legumes such as faba bean.

Among the numerous environmental constrains affecting crop yield, drought is considered the most limiting factor with important economic consequences (Jones, 2004). *M. truncatula* is quite a drought-tolerant plant species compared to grain legumes such as pea (González et al., 1998; Gálvez et al., 2005) or soybean (González et al., 1995). Based on physiological and biochemical studies, *M. truncatula* responses to drought appear to be similar to those described in alfalfa (Rubio et al., 2002; Naya et al., 2007). The relative drought tolerance of *M. truncatula* has been shown in a recent study, where moderate water deficit had only a slight significant effect on plant biomass, presenting some differences among cultivars/ecotypes (Limami et al., 2006).

Nunes et al. (2008) have further corroborated this relative tolerance, showing that under mild drought conditions *M. truncatula* plants were able to avoid leaf dehydration and under severe drought stress plants maintained significantly high net CO_2 fixation rates.

Particular emphasis has been laid on the regulation of symbiotic nitrogen fixation (NF) under drought stress in nodulated legumes. In contrast to earlier studies in soybean (González et al., 1995) and pea (González et al., 1998; Gálvez et al., 2005), analysis in *M. truncatula* suggests that the drought-induced downregulation of sucrose synthase is not the main responsible for the inhibition of NF (R. Ladrera, E.M. González, C. Arrese-Igor, unpublished), similarly to observations in *M. sativa* (Naya et al., 2007). Additionally, the response to drought at the nodule level has been recently analysed under a proteomic perspective (Larrainzar et al., 2007), where new marker enzymes such as plant methionine synthase and bacteroid serine hydroxymethyltransferase were identified. Regarding *L. japonicus*, Díaz et al. (2005) reported an accumulation of proline and oxidative damage in leaves upon different water deprivation treatments. Although the first studies analysing the response of this legume to water deficit have started to emerge, most of the published work so far is based on other *Lotus* spp. (Olsson et al., 1996; Carter et al., 1997; Borsani et al., 1999; 2001; Banon et al., 2004).

Plant responses to salt stress have been extensively analysed (reviewed in Hasegawa et al., 2000; Yamaguchi and Blumwald, 2005), with an especial emphasis on the role played by different osmolytes in homeostasis maintenance. Some compounds such as proline-betaine, trehalose or trigonelline, a pyridine betaine, have been reported to play a role in the response to salt stress of different legumes (Tramontano and Jouve, 1997; Trinchant et al., 2004; López et al., 2008). Furthermore, proline accumulation has been shown to enhance NF during salt stress in *M. truncatula* (Armengaud et al., 2004; Verdoy et al., 2006). In a recent functional analysis, Sanchez et al. (2008) reported a

general increase in the steady-state level of many amino acids, sugars and polyols, with a concurrent decrease in most organic acids in response to gradual salt stress in *L. japonicus* leaves. On the other hand, molecular approaches have been applied to examine the response of *M. truncatula* and *M. sativa* under salinity leading to the identification of several transcription factors related to the plant root response to salt stress (Merchan et al., 2003; de Lorenzo et al., 2007; Merchan et al., 2007).

The effect of low temperatures on plants has also received considerable attention. Unfortunately, little is know about the response of legumes to this type of stress, as most of the published reports are based on model plants such as *A. thaliana*. Plant cold acclimation is a complex process, which involves the specific expression of cold-induced genes to stabilize membranes against freeze-induced injury. This group includes genes encoding late embryogenesis-abundant proteins, enzymes required for osmolyte biosynthesis, antifreeze proteins, chaperones and detoxification enzymes, under the control of several cold-induced transcription factors (Thomashow, 1999; Heino and Palva, 2003). Based on the information available, it appears that *M. truncatula* exhibit a poor freezing tolerance, when compared to other annual legumes (Brandsaeter et al., 2002). This might be due to an ineffective cold acclimation process and low starch reserves in this species (Hekneby et al., 2006). Interestingly, the *M. truncatula* ZFP1 gene, encoding a root-enhanced zinc finger protein with high similarity to a soybean cold-inducible protein, is not regulated by low temperature, suggesting a different physiological function of this protein in both legume species (Xu and Ma, 2004). Some promising results for low temperature legume breeding have been obtained by transgenic expression of an iron-superoxide dismutase in alfalfa, resulting in an enhanced winter survival (McKersie et al., 1993; 2000)

Flooding is another environmental stress that negatively influences germination, seedling establishment and plant development, as it causes a limitation in the flux of oxygen to support plant respiration (Bailey-Serres and Voesenek, 2008). Besides the activation of alcohol and lactic fermentative pathways, flooding stress on *M. truncatula* seedlings induces activity of mitochondrial alanine aminotransferase and glutamate dehydrogenase which may contribute to the maintenance of the redox balance during fermentative growth (Ricoult et al., 2005; 2006). The involvement of non-symbiotic hemoglobins in flooding stress adaptation has been shown in *L. japonicus* (Shimoda et al., 2005), and soybean (Lee et al., 2004), whereas promoter analysis carried out in faba bean suggested that symbiotic leghemoglobins were not induced upon hypoxia (Vieweg et al., 2004).

In the context of GLIP European project, abiotic stress tolerance has been focused to species such as *M. truncatula*, pea and chickpea (Gálvez et al., 2005; de Lorenzo et al., 2007; Larrainzar et al., 2007; Merchan et al., 2007; Marino et al., 2008) leading to identification of factors potentially involved in abiotic stress adaptation and tolerance. The involvement of some genes in abiotic stress response has been already analysed in different legumes. For instance, alfalfa over-expressing chloroplastic MnSOD showed lower cold-induced membrane injuries (McKersie et al., 1996), although these transgenic lines did not present better tolerance to drought stress (Rubio et al., 2002). The transcriptional regulator, *Alfin1*, over-expressed in alfalfa was shown to regulate endogenous NaCl-inducible gene expression, resulting in salinity tolerance (Winicov and Bastola, 1999). Similarly, a drought-responsive AP2-type transcription factor induced several wax-related genes resulting in increased drought tolerance when over-expressed in alfalfa (Aharoni et al., 2004; Zhang et al., 2005). In addition, the stress-

inducible expression of AtDREB1A increases transpiration efficiency in peanut under water-limiting conditions (Bhatnagar-Mathur et al., 2007).

5.4. Breeding for nitrogen fixation

5.4.1. Induction of legume root nodules

Nodulation is initiated by plant roots exuding flavonoid molecules into the soil (Ferguson and Mathesius, 2003). This attracts rhizobia to the roots and concomitantly stimulates them to synthesize a lipochito-oligosaccharide signaling molecule called Nod Factor (NF) (Caetano-Anollés and Gresshoff, 1991; Stacey et al., 2006; Oldroyd, 2007). Using the model species *L. japonicus* and *M. truncatula*, and a predominantly mutant-based approach, many of the genes required for nodule development have now been elucidated (Stacey et al., 2006; see Fig. 2A). This includes genes encoding transmembrane LysM-type receptor kinases believed to be required for NF perception: *LjNFR1* and *LjNFR5* in *L. japonicus*, and *MtNFP*, *MtLYK3* and *LYK4* in *M. truncatula* (Ben Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). Subsequent to perception, NF signaling continues through a NBS-LRR receptor kinase, called *LjSYMRK/MtDMI2* (Endre et al., 2002; Stracke et al., 2002). The signalling cascade then progresses via a number of genes, including those encoding potential potassium ion channels, *MtDMI1*, *LjCASTOR* and *LjPOLLUX* (Ané et al., 2004; Imaizumi-Anraku et al., 2005), putative nucleoporins, *LjNUP133* and *LjNUP85* (Kanamori et al., 2006; Saito et al., 2007), a calcium–calmodulin-dependent protein kinase, *MtCCaMK* (Lévy et al., 2004; Mitra et al., 2004), a cytokinin receptor, *LjLHK1*/*MtCRE1* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Oldroyd, 2007; Tirichine et al., 2007) and finally transcription factors, including *MtNSP1*, *MtNSP2*, *MtERF* and *LjNIN* (Schauser et al., 1999; Kaló et al., 2005; Smit et al., 2005; Middleton

et al., 2007). These genes are all required for nodulation; the loss of any results in reduced, or a complete lack of nodule formation.

5.4.2. Control of legume nodulation

Additional external and internal factors act as negative regulators of nodulation. Mutants unable to synthesize or perceive these factors exhibit increased nodule numbers. The best known of these factors function in the plant's Autoregulation Of Nodulation (AON) pathway (Caetano-Anollés and Gresshoff, 1991; Gresshoff, 2003; Kinkema et al., 2006; see Fig. 2B). This pathway involves long-distance root-shoot signalling initiated during nodule development by the synthesis of a root-derived signal. Grafting experiments (Delves et al., 1986; Jiang and Gresshoff, 1997) have shown this signal (named 'Q') travels to the shoot where it, or a product of its action, is perceived by a LRR receptor kinase, called *GmNARK*/*LjHAR1/MtSUNN* (Krusell et al., 2002; Men et al., 2002; Nishimura et al., 2002a; Searle et al., 2003; Schnabel et al., 2005). Grafting studies have also shown that the gene, *LjKLAVIER*, has a shoot-specific role in AON (Oka-Kira et al., 2005), but the identity of this gene remains unknown. Following perception in the shoot, a novel shoot-derived inhibitor (named 'SDI') is synthesized and travels back down to the roots where it acts to inhibit further nodulation events (Gresshoff and Delves, 1986). Gene chip and real time PCR analysis of leaves from inoculated or uninoculated soybean plants differing in *GmNARK* function, revealed a novel regulation of the octodecanoid pathway, suggesting jasmonic acid signaling is involved in AON (Kinkema and Gresshoff, 2008).

Root-specific AON genes have been identified in pea, *PsNOD3* (Postma et al., 1988), and *M. truncatula*, *MtRdn1* (J. Frugoli, personal communication), that are possibly involved in Q biosynthesis or translocation or SDI perception in the root.

Genes homologous to those detailed above could be identified in faba bean mutant collections (Duc and Picard, 1986; Duc, 1995), potentially leading to improved symbiosis in crop lines.

Other factors that reduce nodule numbers include ethylene and nitrate (Carroll et al., 1985a; 1985b; Ligero et al., 1991; Lee and Larue, 1992; Ferguson and Mathesius, 2003; Ferguson et al., 2005). Mutations that disrupt the plant's ability to perceive these factors alleviate their inhibitory nature, resulting in increased nodule numbers (cf. Penmetsa and Cook, 1997). This includes genes required for ethylene sensitivity and response, such as *LjETR1* and *LjEIN2/MtEIN2* (e.g. Penmetsa et al., 2008). In addition, *nitrate-tolerant symbiosis* (*nts*) mutants that form many nodules when grown under inhibitory nitrate levels have been isolated in soybean and pea (Jacobsen and Feenstra, 1984; Carroll et al., 1985a; 1985b; Delves et al., 1986; Duc and Messager, 1989), but *nts* genes not involved in AON remain to be cloned. Interestingly, the gene *LjASTRAY*, which encodes a bZIP transcription factor with a RING-finger motif, regulates light and photomorphogenic signalling and also nodulation, as loss-of-function mutant exhibit increased nodule number (Nishimura et al., 2002b). Understanding the roles of the above-mentioned regulatory genes will enable optimizing the symbiosis, resulting in tremendous agronomic impacts for faba bean.

5.4.3. Molecular genetics of nodulation and nitrogen fixation in faba bean

Faba bean forms indeterminate root nodules with the soil bacteria, *Rhizobium leguminosarum* bv. *viciae*, and in many regions where effective rhizobia populations are present, field inoculation is not practiced. Biodiversity in host-microbe populations has been exploited to improve nitrogen fixation rates of faba bean (e.g. Mytton et al., 1977; Mytton, 1984; Knaak et al., 1993). Recent molecular insights, including the

identification of symbiotic genes in *L. japonicus* and *M. truncatula*, will enhance these classical breeding approaches. The use of faba bean mutants that fail to nodulate (Nod^-), excessively nodulate (Nod^{++}) even in the presence of nitrate (*nts*), or are non-functional (Fix^-, i.e., fail to fix nitrogen), including those spontaneously occurring (*sym-1*; Duc and Picard, 1986; Haser et al., 1992) or chemically induced (Duc, 1995), offer further potential in this area. Similarly, the identity of numerous faba bean genes encoding unknown nodule proteins (nodulins) and leghemoglobins (required for nitrogen fixation) (Perlick and Pühler, 1993; Frühling et al., 1997; Schröder et al., 1997; Hohnjec et al., 2000; Vieweg et al., 2004) should aid in efforts to improve nitrogen fixation *via* coupling transgenic techniques with classical breeding methods.

5.4.4. Application of functional genomics in faba bean

Advances in genomic technology and insights could aid nodulation and nitrogen fixation research in faba bean. Following similar work in cereals, it was discovered that legume genome maps share broad similarity (called macro-synteny; Choi et al., 2004b). Likewise, molecular markers in legume genomes (usually ESTs reflecting biochemical functions) are found in similar chromosomal blocks. Thus, discovery of markers linked to a certain phenotype, for instance, in *M. truncatula* may provide a tool to identify the same characteristic in the otherwise unexplored faba bean. It therefore becomes critical that molecular linkage maps of faba bean include both ESTs and phenotypes (including QTLs) relating to nodulation, nitrogen use efficiency and nitrogen fixation, and that variation for these phenotypes is mapped to such conserved EST markers. The recently completed, and near-completed, genome sequences of *G. max, M. truncatula* and *L. japonicus* respectively, will greatly aid in this area of research

(http://www.phytozome.net/soybean; http://www.medicago.org/genome/; Sato et al., 2008).

6. Concluding Remarks

The use of model legumes to investigate important grain legume traits has already improved our knowledge on legume biology. In particular it allows important breakthroughs in our understanding of nitrogen-fixing symbiosis, and begins to bring clues to legume seed quality and resistance to biotic and abiotic stresses. The high level of synteny and conservation that exist between most legume genomes should allow an efficient transfer of all the knowledge that is being accumulated in these model legumes to improve faba bean, a grain legume for which its large genome size limits the development of post-genomic tools. Indeed, candidate genes identified by transcriptomic, proteomic or map-based cloning can be transferred to elite cultivars of faba bean after validation of its function by MAS or genetic transformation.

While the use of model legumes has already increased our knowledge on several important aspects of grain legumes, many gaps remain in our understanding of legume quality, nitrogen-fixing capacities and resistance to stresses. In parallel, biotechnological improvements allow the development of different post-genomic tools to facilitate the identification of genes and pathways, functional analysis of these genes and the search for favourable alleles in germplasm collections. Although these tools are expected to greatly help the transfer of important genes for crop improvement in a near future, they are likely to be still insufficient. For the improvement of any trait, an integration of knowledge coming from molecular biologists, plant physiologists, plant pathologists, agronomists, applied breeders and experts on social-environmental impact, involving multi-criteria decision-making programs, is required. Within the framework

of the above mentioned GLIP project some advances have been made possible. However, for real results "at the fork level", the recently created interdisciplinary research networks, and others yet to come, need to be continued, which will require extended commitment from funding agencies at the national and international levels.

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Tables

Table 1. Comparison of genome size, seed composition and available resources between *Vicia faba*, *Pisum sativum* and *Medicago truncatula*. Phylogeny for the species according to Choi *et al.* (2004a) is represented as a tree on the left.

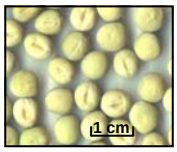
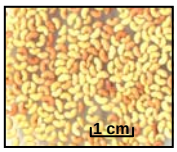
Species	Genome size (Mb)	Genomic resources * NCBI, release 31 July 2008	Genetic resources	QTL mapping	Post-genomic resources *NCBI, release 31 July 2008	Seed composition
Vicia faba **Tribe *Viceae***	**~13,000** (2n = 12 chrom.)	**466 core nucleotide sequences** * **512 genome survey sequences***	**Mapping populations and linkage maps:** ▪ ~20 F2 populations ▪ 14 major linkage groups Vaz Patto *et al.* 1999; Ávila *et al.* 2003, 2004; Román *et al.* 2004 and references therein **_Vicia faba_ germplasm collections at:** ▪ http://193.50.15.18/legumbase/ ▪ http://www.icarda.cgiar.org/	**QTL mapped for seed weight and disease resistance** Vaz Patto *et al.* 1999; Ávila *et al.* 2004; Román *et al.* 2002, 2003	<10 EST*	 30% proteins 42% starch 2% lipids 9% cellulose 4% soluble sugars (Duc et al., 1999)
Pisum sativum **Tribe *Viceae***	**~5,000** (2n=14 chrom.)	**3,864 core nucleotide sequences*** **154 genome survey sequences*** **BAC libraries:** ▪ **Line PI269818:** ~3 genome equivalents (Coyne et al., 2007) ▪ **Cv. Caméor:** ~10 genome equivalents (A. Bendahmane, personal communication)	**Mapping populations and linkage maps:** ▪ ~20 population (F2, F5 to F9) ▪ 7 linkage groups (Weeden et al., 1998 ; Ellis and Poyser 2002 ; Tar'an et al., 2003; Loridon et al., 2005 ; Pilet-Nayel et al., 2005 ; Aubert et al., 2006) **Pea germplasm collections:** ▪ http://193.50.15.18/legumbase/ ▪ http://www.jic.ac.uk/germplas/pisum/; www.ars-grin.gov/ **Mutant populations:** TILLING (Dalmais et al., 2008)	**QTL mapped for a range of agronomic traits** e.g. seed traits, disease resistance, frost tolerance (Tar'an et al., 2004; Pilet-Nayel et al., 2005; Timmermann-Vaughan et al., 2005; Burstin et al., 2007; Prioul-Gervais et al., 2007; Lejeune-Hénaut et al., 2008)	**EST count :** 6,327 ESTs* (580 from seed cDNA libraries) **Transcriptomics :** Ps6kOLI1 microarrays (Weigelt et al., 2008) **Proteome maps :** e.g. leaves (Schiltz et al., 2004), mature seeds (Bourgeois et al., 2008), and organelles (Bardel et al., 2002 ; Phinney and Thelen 2005)	 23% proteins 50% starch 2% lipids 7% cellulose 5% soluble sugars (Bastianelli et al., 1998)
Medicago truncatula **Tribe *Trifolieae***	**~500** (2n=16 chrom.)	**6,149 core nucleotide sequences** * **168,815 genome survey sequences*** **BAC libraries:** 5 to 20 genome equivalents (Nam et al., 1999; Choi et al., 2004a) **Sequencing** gene-rich regions http://www.medicago.org/genome/	**Mapping populations and linkage maps:** ▪ ~800 populations, including five F6 to F7 populations of recombinant inbred lines ▪ 8 linkage groups (Thoquet et al., 2002 ; Green et al., 2006) ***M. truncatula* germplasm collections:** (Greene et al., 2006) **Mutant populations:** ▪ TILLING (Thompson *et al.* 2005), ▪ *Tnt1* insertion (Tadege *et al.* 2008)	**QTL mapped for aerial morphogenesis, including flowering, resistance to disease, spring black stem and leaf spot** (Julier et al., 2007 ; Vailleau et al., 2007 ; Ameline-Torregrosa et al., 2008; Kamphuis et al., 2008 ; Pierre et al., 2008)	**EST count:** 249,625 ESTs* (12,937 from seed cDNA libraries) **Transcriptomics :** Mt16kOLI1 microarrays (Hohnjec et al., 2005) Affymetrix Medicago GeneChip (Gene expression atlas from Benedito et al., 2008) **Proteome maps from various tissues, including seeds:** (Watson et al., 2003)	 46% proteins 1% starch 13% lipids 10% cellulose 5% soluble sugars (Duc, 2004)

Figure legends

Figure 1. Large-scale synteny blocks between *M. truncatula* (Mt) and *L. japonicus* (Lj) chromosomes. Synteny was detected along entire chromosomal segments in the case of *M. truncatula* chr 1 and *L. japonicus* chr 5, while rearrangements of colinear blocks in other chromosomal regions resulted in a difference of chromosome number between the two species. Bars with the same color or pattern show homologous chromosomal regions and arrows in the boxes indicate the orientation of the chromosomes (short arm – long arm). Blank boxes represent genomic regions where the correspondence between the chromosomes has not yet been revealed clearly.

Figure 2. Signaling of nodulation. **A**. Nodulation factor perception leads to infection and cell division in legume roots. NF signal transduction occurs through four biochemical stages, beginning with receptor kinases, then membrane channels causing calcium oscillations, followed by a protein kinase modulator (CCaMK) and a cytokinin receptor, and finally through transcription factor control of diverse genetic pathways. This cascade occurs in different cell layers of the root. For example, NF perception likely occurs primarily in the root hair and the epidermis, perhaps fractionally so in the cortex. Note that epidermal and root hair cells do not divide and hence there is a need for a secondary signal to travel to the root interior and the pericycle, the target tissue for nodule growth. Later parts of the cascade involving transcription factors may be localized to this region. Detailed spatial analysis using gene fusions and reporter genes is needed to elucidate such events. **B**. Autoregulation of Nodulation (AON) controls nodule number by a systemic regulatory circuit involving root-shoot communication. NF perception leads to an activated state closely associated with the induction of cell divisions. This process may be mimicked by mycorrhizal fungi, which undergo

eukaryotic cell cycle progression similar to that of plant cells (cf., Meixner et al, 2007). This activated state is 'reinforced' by successful invasion and nitrogen fixation. The mobile 'Q' signal is perceived by leaf tissues to release a putative ligand peptide that interacts with the NARK receptor kinase in the phloem parenchyma of the leaf. NARK is known to down-regulate several genes in the octadecanoid pathway leading to the synthesis of jasmonic acid (JA). Metabolites related to this pathway or parallel targets of NARK, including 'SDI', are presumed to migrate via the phloem to the developing nodule primordium, blocking further nodule development. In temperate legumes, such as pea and *M. truncatula*, this blockage occurs earlier than in the determinate-nodulating soybean (Mathews et al., 1989).

Figure 1

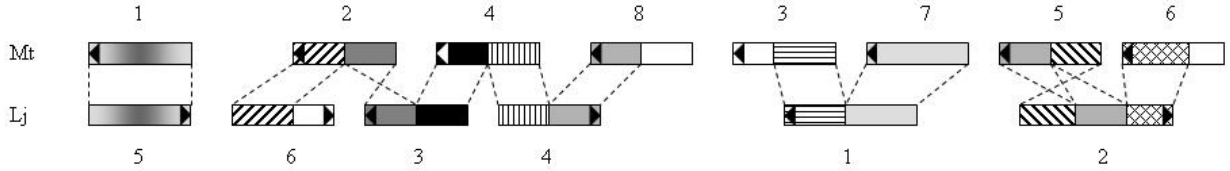
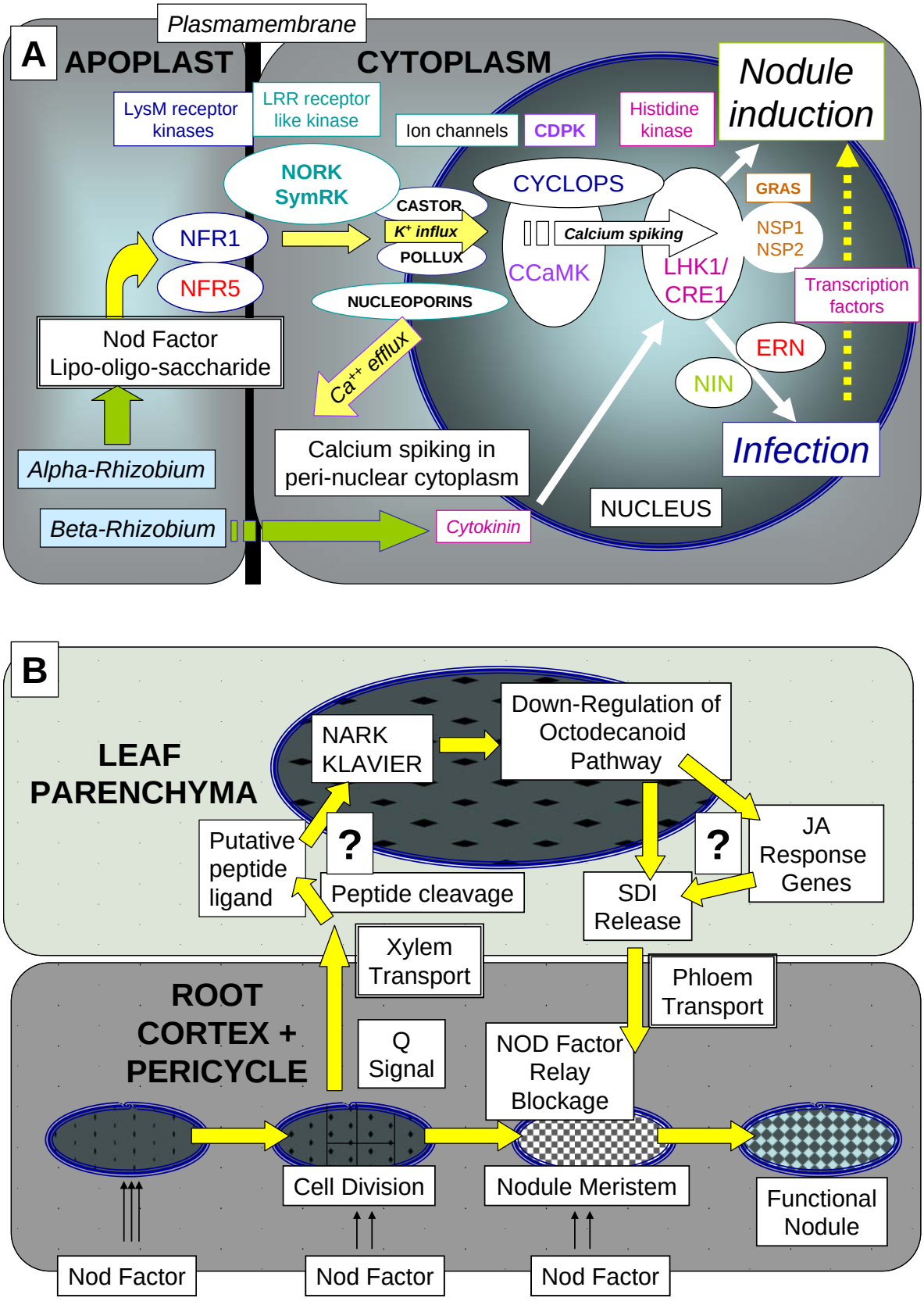


Figure 2


A
Plasmamembrane
APOPLAST
CYTOPLASM
LysM receptor kinases
LRR receptor like kinase
Ion channels
CDPK
Histidine kinase
Nodule induction
NORK SymRK
CASTOR
K+ influx
POLLUX
CYCLOPS
Calcium spiking
GRAS
NSP1 NSP2
NFR1
NFR5
CCaMK
LHK1/ CRE1
Transcription factors
NUCLEOPORINS
Nod Factor Lipo-oligo-saccharide
Ca++ efflux
ERN
NIN
Infection
Alpha-Rhizobium
Calcium spiking in peri-nuclear cytoplasm
NUCLEUS
Beta-Rhizobium
Cytokinin
B
LEAF PARENCHYMA
NARK KLAVIER
Down-Regulation of Octodecanoid Pathway
Putative peptide ligand
?
Peptide cleavage
?
JA Response Genes
SDI Release
Xylem Transport
Phloem Transport
ROOT CORTEX + PERICYCLE
Q Signal
NOD Factor Relay Blockage
Cell Division
Nodule Meristem
Functional Nodule
Nod Factor
Nod Factor
Nod Factor